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Keywords
Ovotoxicant, follicle, steroidogenesis

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Impact of environmental exposures on ovarian function and role of xenobiotic metabolism during ovotoxicity

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Abstract

The mammalian ovary is a heterogeneous organ, and contains oocyte-containing follicles at varying stages of development. The most immature follicular stage, the primordial follicle, comprises the ovarian reserve and is a finite number, defined at the time of birth. Depletion of all follicles within the ovary leads to reproductive senescence, known as menopause. A number of chemical classes can destroy follicles thus hastening entry into the menopausal state. The ovarian response to chemical exposure can determine the extent of ovotoxicity that occurs. Enzymes capable of bioactivating as well as detoxifying xenobiotics are expressed in the ovary and their impact on ovotoxicity have been partially characterized for trichloroethylene, 7,12-dimethylbenz[a]anthracene, and 4-vinylcyclohexene. This review will discuss those studies, as well as illustrate where knowledge gaps remain for chemicals that have also been established as ovotoxicants.

Keywords

Ovotoxicant; follicle; steroidogenesis

Introduction

The ovary is the female gonad with two major functions, production of the germ cell and the sex steroid hormones, chiefly 17β-estradiol (E_2) and progesterone (P_4). At birth, the ovary contains a finite number of primordial follicles, comprised of a meiotically-arrested oocyte surrounded by squamous granulosa cells (Hirshfield, 1991). These follicles comprise the ovarian reserve from which pre-ovulatory follicles are developed. A number of chemical classes can deplete ovarian follicles and alter steroidogenesis leading to impaired ovarian function and infertility, including but not limited to, environmental, industrial, chemotherapeutic and xenoestrogenic chemicals (Hoyer and Sipes, 1996). This review article will describe studies that have investigated the impact of ovarian metabolism on ovotoxicity induced by xenobiotics. Three chemicals for which the majority of knowledge on the impact of ovarian metabolism is available will be first described – Trichloroethylene (TCE), 7,12-dimethylbenz[a]anthracene (DMBA) and 4-vinylcyclohexene (VCH). The

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There are no conflicts of interest.

remainder of this review will summarize the ovarian effects of chemicals for which less is known on the role of metabolism, and will indicate where knowledge gaps remain.

Biotransformation enzymes

Of the enzymes capable of biotransformation of chemical compounds, certain enzymes that have emerged as having roles in ovarian metabolism are discussed in this review. A brief summary of their function is provided here as an introduction to those studies in which they are found to have roles in determining the extent of ovotoxicity that occurs.

The cytochrome P450 (CYP) enzymes comprise a large family that play critical roles in phase I metabolism of a wide variety of xenobiotics. CYP enzymes are found in all tissues and they can be involved in activation or detoxification, depending on the chemical substrate (Casarett and Doull, 2008). Another group of phase I biotransformation enzymes are the epoxide hydrolases (EH) which catalyze the addition of water to alkene epoxides and arene oxides. There are five forms of EH, including the isoform under discussion in this review, microsomal epoxide hydrolase (mEH). mEH has a wide alkene epoxide and arene oxide substrate range, but has a preference for mono-substituted epoxide structures (Casarett and Doull, 2008).

Glutathione (GSH) is a ubiquitous antioxidant compound, present in all cells, and is composed of glycine, cysteine and glutamic acid. Formation of GSH occurs in two steps catalyzed by γ-glutamylcysteine (γ-Gcl) and glutathione synthetase (Gss). Conjugation of GSH to a chemical generally represents a phase II detoxification modification. GSH xenobiotic conjugation is catalyzed by the glutathione *S*-transferase (Gst) family of enzymes, which include the isoforms alpha, pi, mu, omega and theta, and comprise about 10% of total cellular protein (Casarett and Doull, 2008).

Trichloroethylene

TCE is a common water contaminant (Davidson and Beliles, 1991). Its lipophilic characteristic together with low boiling point makes it ideal for several industrial processes including metal degreasing and dry cleaning (Weiss, 1996). Numerous commercially used products like wood stains, adhesives, lubricants and paint removers contain TCE (Wu and Berger, 2007). Due to widespread use, human exposure can occur through drinking, inhalation or transdermal absorption (EPA, 1985) and U.S. urban areas have approximately three times more detectable TCE compared to rural areas (Wu and Schaum, 2000).

Female rats exposed to TCE via inhalation (1700 ppm, 2h/d, 5 d/wk) display decreased oocyte fertilizability induced by alterations to the oocyte plasma membrane composition (Berger and Horner, 2003). Oocytes from female rats exposed to TCE orally (0.45% TCE in 3% Tween) for 4, 5, or 14 days also showed less capacity for binding of and fertilization by unexposed rat sperm compared to oocytes from vehicle control exposed females (Wu and Berger, 2007) (Figure 1).

Hepatic TCE metabolism primarily occurs in two ways. The first is through CYP enzyme-mediated oxidation (Nakajima *et al.*, 1988; Guengerich *et al.*, 1991a), resulting in formation of trichloroethanol (TCOH), which can further be metabolized to TCOH glucuronide (Cummings and Lash, 2000). TCE can also be metabolized to trichloroacetic acid (TCAA), dichloroacetic acid (DCA) and monochloroacetic acid (Cummings and Lash, 2000). The CYP isoforms involved in TCE metabolism are CYP1A1/2, CYP2B1/2, CYP2C11/6 and CYP2E1 (Nakajima *et al.*, 1988; Nakajima *et al.*, 1990; Guengerich *et al.*, 1991a; Guengerich and Turvy, 1991b; Nakajima *et al.*, 1992a; Nakajima *et al.*, 1992b; Nakajima *et al.*, 1993). Of these isoforms CYP2E1 has the highest affinity for TCE (Nakajima *et al.*, 1990; Guengerich *et al.*, 1991a; Guengerich and Turvy, 1991b; Cummings and Lash, 2000).

In vitro exposure of mouse oocytes to TCAA, DCA and TCOH also reduce fertilization rates (Cosby and Dukelow, 1992) and have been detected in the rat ovary following TCE exposure through drinking water (0.45% for 2 wk; Wu and Berger, 2007). Additionally, increased ovarian CYP2E1 activity was demonstrated in these rats suggesting ovarian TCE metabolism through the CYP-dependent oxidative pathway (Reinke and Moyer, 1985).

In extra-ovarian tissues, the second route of TCE metabolism of TCE is through GSH conjugation resulting in S-(1,2-dichlorovinyl)glutathione (DCVG) formation (Lash *et al.*, 1995; Lash *et al.*, 1998). Dipeptidase and γ–glutamyltransferase (GTT) further metabolize DCVG to *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC). This product can then be metabolized either by cysteine *S*-conjugate *N*-acetyl-*S*-transferase (NAT) to form *N*-acetyl-DCVC (NAcDCVC). DCVC can also be converted by the cysteine conjugate β–lyase (β-lyase) to form a reactive thiol compound (Lash *et al.*, 1995; Lash *et al.*, 1998). NAcDCVC has been detected in both rats and human urine after TCE exposure (Commandeur and Vermeulen, 1990; Birner *et al.*, 1993). *In vitro* exposure of rat oocytes to DCVC (5 mM; 4 h) reduced zona pellucida-free oocyte fertilizability (Wu and Berger, 2008), which is interesting since GSH conjugation usually results in less reactivity compared to the parent compound. In summary, TCE induces ovotoxicity through bioactivation by the CYP-dependent and GSH-conjugating pathways: whether these biotransformation events occur in ovarian tissue, however, remains unclear.

7,12-dimethylbenz(a)anthracene

7,12-dimethylbenz(a)anthracene (DMBA) is a polycyclic aromatic hydrocarbon (PAH) and is a model carcinogenic chemical for its ability to induce ovarian (Kanter *et al.*, 2006), skin (Diagaradjane *et al.*, 2006) and mammary (Russo and Russo, 1996) tumors in rodents. Humans are exposed to this chemical through burning of organic materials, thus exposure can come from cigarette smoke and car exhaust fumes (Lawther and Waller, 1976). DMBA destroys all follicle stages including corpora lutea in a dose-dependent manner in mice and rats (Mattison, 1980; Hoyer, 2001; Rajapaksa *et al.*, 2007a; Igawa *et al.*, 2009). A decrease in ovarian volume typically results from lack of ovarian follicles (Mattison and Schulman, 1980; Weitzman *et al.*, 1992).

DMBA is metabolized to DMBA-3,4-epoxide by CYP1B1, which is hydrolyzed to DMBA-3,4-diol by the action of mEH. DMBA-3,4-diol then undergoes epoxidation by CYP1A1 or CYP1B1 to form the ultimate ovotoxic and carcinogenic compound, DMBA-3,4-diol-1,2-epoxide (Miyata *et al.*, 1999). CYP1A1, CYP1B1 and mEH enzymes are induced at mRNA and protein levels in mouse and rat ovaries exposed to DMBA (Cannady *et al.*, 2002; Shimada *et al.*, 2003; Rajapaksa *et al.*, 2007a; Igawa *et al.*, 2009). In cultured post natal day 4 (PND4) rat ovaries meh mRNA and protein are increased during DMBA (1 μM) exposure at a time point prior to follicle depletion (Rajapaksa *et al.*, 2007b; Igawa *et al.*, 2009). Use of the competitive mEH inhibitor, cyclohexene oxide prevented DMBA-induced primordial follicle loss, however, the DMBA-3,4-diol metabolite was unaffected by mEH inhibition (Figure 2). These results support that mEH is involved in ovarian DMBA bioactivation (Rajapaksa *et al.*, 2007b; Igawa *et al.*, 2009).

Cultured PND4 mouse ovarian primordial follicle oocytes have increased expression of the pro-apoptotic protein, BAX, following DMBA (1μM) exposure, mediated through the action of the aryl hydrocarbon receptor (AHR; Matikainen *et al.*, 2001). Use of an AHR antagonist, alpha-naphthoflavone (ANF) prevented BAX-induced follicle loss caused by DMBA (Matikainen *et al.*, 2001). Also, intra-ovarian ANF treatment (80mg/kg) during DMBA exposure (10 μg) prevented follicle destruction in mice (Shiromizu and Mattison, 1985). Further, *Bax*- and *Ahr*-deficient mice are resistant to DMBA-induced primordial follicle destruction (Matikainen *et al.*, 2001). Although the mechanisms behind protection

are not understood, DMBA-induced oocyte destruction is preventable by co-treatment with the E_2 agonist Tamoxifen (TAM) *in vivo* (Ting and Petroff, 2010).

While DMBA bioactivation is at least partially characterized, less is known about DMBA detoxification. GSH supplementation (glutathione ethyl ester; GEE; 5 mM) protects ovaries from DMBA-induced follicle loss in cultured pre-ovulatory follicles from Sprague-Dawley rats (Tsai-Turton *et al.*, 2007a). Also, *Gstp*-null mice have increased DMBA-induced skin tumor formation compared to wild type littermates (Henderson *et al.*, 1998). PND4 cultured rat ovaries exposed to DMBA (1 μM) have increased Gstp mRNA and protein expression (Bhattacharya *et al.*, 2012) at a time-point prior to DMBA-induced follicle loss (Igawa *et al.*, 2009). The increased GSTP is associated with negative regulation of pro-apoptotic c-Jun N-terminal kinase (JNK), indicating a protective role for GSTP within the ovary (Bhattacharya and Keating, 2012). Furthermore, DMBA exposure increased Ahr mRNA and protein, and Nuclear factor erythroid-related factor 2 (Nrf2) protein suggesting that these transcription factors play role(s) in ovarian xenobiotic biotransformation enzyme activation during DMBA exposure (Bhattacharya and Keating, 2012).

PND4 mouse ovaries exposed to DMBA (50 nM) had altered mRNA expression of a number of genes involved in primordial follicle activation, cell survival and proliferation (Sobinoff *et al.*, 2011). One pathway that was identified was the phosphatidylinositol-3 kinase (PI3K) pathway. PI3K is essential for oocyte viability as well as regulation of the rate by which primordial follicles are recruited into the growing follicular pool (Castrillon *et al.*, 2003; Reddy *et al.*, 2005; Liu *et al.*, 2006). Activation of this pathway occurs when granulosa-expressed Kit Ligand (KITL) binds to the oocyte-expressed receptor, c-KIT (Manova *et al.*, 1990; Orr-Urtreger *et al.*, 1990; Horie *et al.*, 1991). c-KIT undergoes auto-phosphorylation and the PI3K components are activated (Serve *et al.*, 1994). PI3K converts PIP2 to PIP3, which acts as a second messenger eventually resulting in phosphorylation (activation) of AKT (Engelman, 2009). Activated AKT can promote expression of a number of pro-survival genes, and can also negatively regulate pro-apoptotic genes, including the Forkhead transcription factor isoform 3a (*Foxo3a*; Brunet *et al.*, 1999). Increased AKT phosphorylation with a concomitant decrease in FOXO3A phosphorylation, along with activation of the PI3K-regulated protein, mammalian target of rapamycin (mTOR) was observed in DMBA-treated primordial follicle oocytes (Sobinoff *et al.*, 2011). Inhibition of PI3K signaling accelerated DMBA-induced loss of all follicle stages in cultured PND4 rat ovaries (Keating *et al.*, 2009), thus a role for PI3K signaling during DMBA-induced ovotoxicity is supported.

4-Vinylcyclohexene

4-vinylcyclohexene (VCH) is produced as a by-product of the pesticide, rubber, plastic and flame retardant industries (Rappaport and Fraser, 1977). Human exposures are through dermal contact, oral intake (NTP, 1989) and inhalation (Bevan *et al.*, 1996). VCD selectively destroys primordial and small primary follicles leading to premature ovarian failure in mice and rats (Smith *et al.*, 1990; Hooser *et al.*, 1994; Mayer *et al.*, 2002).

VCH is bioactivated in the ovary to the ultimate diepoxide ovotoxic metabolite, VCD, through the action of CYP2E1 (Rajapaksa *et al.*, 2007b). *Cyp2e1*-null mice had less follicle loss relative to their wild type littermates (Rajapaksa *et al.*, 2007b), thus CYP2E1 is involved in VCH bioactivation to VCD. Detoxification of VCD is thought to occur through the action of ovarian expressed mEH. VCD induces meh mRNA and protein expression both *in vivo* (Cannady *et al.*, 2002) and *in vitro* (Keating *et al.*, 2008; Bhattacharya *et al.*, 2012), prior to follicle loss in mice and rats. Inhibition of mEH using CHO in the presence of VCD caused more follicle loss relative to VCD-treated ovaries, supporting a detoxification role for mEH during VCD-induced ovotoxicity (Bhattacharya *et al.*, 2012; Figure 3).

A protective role for the ovarian *Gstp* isoform has also been reported during VCD exposure (Keating *et al.*, 2010). Gstp mRNA and protein are increased in response to VCD, and in a similar manner to that during DMBA-induced ovotoxicity, GSTP forms a protein complex with JNK, and inhibits JNK action, as evidenced by reduced phosphorylation of the JNK target, c-Jun (Keating *et al.*, 2010). GSH conjugates of VCD have also been detected in media from VCD-treated cultured PND4 mouse ovaries (Rajapaksa, 2007), indicating that GSH-conjugation to VCD is a likely detoxification mechanism during VCD exposure, however, the role for GSTP in this conjugation remains unclear.

VCD reduces PI3K signaling in primordial and small primary oocytes (Keating *et al.*, 2011). Decreased phosphorylation of c-KIT (Mark-Kappeler *et al.*, 2011) and oocyte-expressed AKT (Keating *et al.*, 2011) occur rapidly after VCD exposure in PND4 cultured rat ovaries. Additionally, there is a decrease in oocyte FOXO3a in the VCD target follicles (Keating *et al.*, 2011). In an apparent protective response by the ovary, *Kitl* mRNA is up-regulated in response to VCD, and, in fact, exogenous addition of KITL during VCD exposure partially attenuates VCD-induced follicle loss (Fernandez *et al.*, 2008). Further, PI3K inhibition prevents VCD-induced primordial but not small primary follicle loss. It is hypothesized that VCD may accelerate the entry of primordial follicles into the growing pool to replace those small primary follicles destroyed (Keating *et al.*, 2009) through classic apoptotic pathways (Springer *et al.*, 1996a; Springer *et al.*, 1996b; Hu et al, 2001).

An interesting role for PI3K signaling in regulation of xenobiotic metabolism enzyme gene expression is emerging. The ovotoxic outcomes of DMBA and VCD exposures are altered when PI3K is inhibited – follicle loss by DMBA is accelerated, while that of VCD is lessened (Keating *et al.*, 2009). Since mEH bioactivates DMBA but detoxifies VCD, it is logical to consider that altered mEH expression may occur during PI3K inhibition. Furthermore, it is known that the transcription factors C/EBPα and C/EBPβ are responsible for mEH induction through PI3K signaling (Ki and Kim, 2008). It has recently been demonstrated that mEH is indeed increased during PI3K inhibition (Bhattacharya *et al.*, 2012), providing explanation for the ovotoxic outcomes observed with DMBA and VCD when PI3K is inhibited (Figure 4). Also, inhibition of PI3K signaling has been demonstrated to impact expression of AhR, Nrf2, Gstp, Gstm at the mRNA, protein level or both (Bhattacharya and Keating, 2012). New roles for this pathway are emerging due to manipulation of this pathway through chemical means which may allow for future therapeutic interventions.

Methoxychlor

Methoxychlor (MXC) is an organochlorine pesticide and insecticide which was used as a replacement for dichlorodiphenyltrichloroethane (DDT; DHHS., 2002). MXC acts as an endocrine disrupting chemical (EDC) and has several adverse effects on reproductive function in female mice including persistent estrus (Martinez and Swartz, 1991), reduced fertility (Cummings and Gray, 1989), ovarian atrophy (Martinez and Swartz, 1991; Martinez and Swartz, 1992; Eroschenko *et al.*, 1997; Gupta *et al.*, 2006) and dose-dependent follicular atresia (Martinez and Swartz, 1991; Eroschenko *et al.*, 1997). Cultured antral follicles from mice exposed to MXC (100 μg/μl/96 h) demonstrate decreased antral follicle growth and increased antral follicle atresia (Miller *et al.*, 2005; Gupta *et al.*, 2006; Miller *et al.*, 2006).

MXC's role as an EDC has been demonstrated by several *in utero* exposure studies. Pregnant mice exposed to MXC (5.0 mg) via oral gavage from gestation day (GD) 6 to 15 had accelerated vaginal opening in offspring (Swartz and Corkern, 1992), characteristic of an estrogenic effect. Additionally, lipid accretion in ovarian theca and interstitial cells was observed in adult mice exposed orally to MXC (5.0 mg/d/4 wk) which also represent estrogenic effects (Martinez and Swartz, 1992). Conversely, MXC exhibits endocrine

disruption through altered ovarian steroidogenesis. Cultured antral follicles from mice exposed to increasing concentrations of MXC (1–100 μg) over a time course of exposure (24–96 h) had dose-dependent decreased mRNA expression of the steroidogenic enzyme *Hsd3b1* (10 μg MXC - 48 h). At a higher concentration and longer exposure to MXC (100 μg - 96 h), mRNA encoding *Hsd17b1* was also decreased. CYP1B1 enzyme increases metabolism of E_2 and was decreased by MXC exposure (100 μg - 96 h), along with a concomitant decrease in E_2 level (Basavarajappa *et al.*, 2011). Taken together, these data indicate that decreased E_2 may contribute to MXC-induced inhibition of antral follicle growth in mice.

MXC is metabolized to 1,1,1-trichloro-2-(4-hrdroxyphenyl)-2-(4-methoxyphenyl)ethane (mono-OH) through the action of CYP2C9 and CYP1A2 enzymes (Hu and Kupfer, 2002; Hu *et al.*, 2004; Hazai and Kupfer, 2005). Further conversion of mono-OH to 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) is mediated by CYP1A2, CYP2C8, CYP2C19, CYP2D6 and CYP3A4 (Hu and Kupfer, 2002). CYP enzymes (CYP1A1, CYP1B1, CYP1A2, CYP2C29) capable of metabolizing MXC are expressed in mouse ovarian tissues (Symonds *et al.*, 2006). Also CYP2C29 mRNA expression level is increased through an E_2 receptor (ER)-linked mechanism in mouse ovarian surface epithelium following MXC exposure (3 μM/14 d; Symonds et al., 2006).

The MXC metabolite mono-OH decreases E_2 levels through impacts on mRNA encoding enzymes in the steroidogenic pathway (Craig *et al.*, 2010). *Cyp11a1*, *Cyp17a1*, and *Cyp19a1* mRNA were reduced in cultured mouse antral follicles exposed to mono-OH (10 μg - 96 h) with a decrease in E_2 synthesis (Craig *et al.*, 2010). In contrast, HPTE has not been observed to affect mRNA or protein expression of Cyp11a1 and while no impact of HPTE on steroidogenic gene mRNA has been demonstrated, HPTE (50 nM for 24 h) has been shown to inhibit progesterone (P4) formation in cultured rat theca-interstitial and granulosa cells and to decrease the catalytic activity of CYP11A1 (Akgul *et al.*, 2008). Also, luteal cells exposed to HPTE have reduced P4 production (Akgul *et al.*, 2011), however, in this cell type, an inhibitory effect of HPTE on the steroidogenic step catalyzed by CYP11A1 and CYP11A1 activity was observed (Akgul et al., 2011). No impact of HPTE on Cyp11a1 mRNA or protein level was observed however (akgul et al., 2011). Also, lack of any effect of HPTE on *Gpx3*, *Gst*, or Cyp17a1 was observed in vivo in mice (Waters *et al.*, 2001). Moreover, mice dosed with HPTE (32 mg/kg/20 d; intraperitoneally (i.p.)) have increased antral follicle loss but, conversely, increased ovarian surface epithelium proliferation (Borgeest *et al.*, 2002).

2,3,7,8-tetrachlorodibenzo-p-dioxin

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a halogenated aromatic hydrocarbon (HAH) and widespread environmental pollutant (Poland et al., 1982). TCDD is lipophilic and has a slow rate of metabolism and excretion, thus it readily concentrates in the food chain (Safe *et al.*, 1991). TCDD is a by-product of the manufacture of herbicides, insecticides and disinfectants and human exposure comes in the form of waste incineration. Natural sources of TCDD also exist, including forest fire and volcanic eruptions (Ho *et al.*, 2006).

TCDD is a reproductive toxicant (Peterson *et al.*, 1993; Li *et al.*, 1995). Female rats exposed to a single oral dose of TCDD (10 μg/kg) experienced prolonged diestrus stages with concomitant reduced times spent in proestrus and estrus. Also, a reduction in the number of oocytes ovulated due to TCDD has been reported (Li *et al.*, 1995).

Developing ovaries are particularly sensitive to TCDD (Jablonska *et al.*, 2010). Pregnant female Lewis rat dams were exposed to TCDD (50 ng/kg/wk) via oral gavage on both GD 14 and 21 and both PND 7 and 14. Following birth, pups were weaned at PND 21, followed

by exposure to vehicle control or TCDD (50 ng/kg/wk; gavage). Ovaries from the TCDD exposed F1 females were then transplanted into control F1 females and vice versa, followed by TCDD exposure (50 ng/kg/wk; gavage) until sacrifice at 8 months. Both i*n utero* and lactational TCDD exposure induced acyclicity as the females aged (Jablonska *et al.*, 2010). These studies raise concerns over *in utero* TCDD exposure for female reproductive health.

Several studies have shown that TCDD acts as an EDC through inhibition of E_2 production. Chronic TCDD exposure (200 ng/kg/wk) decreased E_2 synthesis and caused irregular cyclicity in the absence of follicle depletion in rats (Franczak *et al.*, 2006; Shi *et al.*, 2007). TCDD exposure (10, 40, 100 ng/kg/14 d; orally) decreased E_2 concentration, impaired follicular development (Heiden *et al.*, 2006), and suppressed *Cyp11a1*, *Cyp19a1a* and *Star* mRNA expression (Heiden *et al.*, 2008) in zebrafish. Additionally, E_2 production was inhibited when human luteinized granulosa cells were exposed *in vitro* to TCDD (10 nM; alternate days; 8 d; Morán *et al.*, 2000).

There is limited information on the impact of ovarian metabolism on TCDD ovotoxicity. A role for GSH conjugation to TCDD as a mechanism of detoxification has been indicated in female rats in which GSTM increased following an oral dose of TCDD (125 ng/Kg/d; Chen *et al.*, 2009).

Bisphenol A

BPA (4,4′-isopropylidenediphenol) is a diphenyl compound consisting of two hydroxyl groups in the “para” position making it very similar to diethylstilbestrol (DES) (Papaconstantinou *et al.*, 2000; Markey *et al.*, 2001). BPA is widely used in the manufacture of polycarbonated plastics, epoxy resins, dental sealants and as a stabilizing reagent in plastic production. Humans are exposed to BPA through the interior coating of food cansmilk containers, food storage vessels, wine storage vats, baby formula bottles, water pipes, automotive lenses, optical lenses, protective coatings, adhesives, protective window glazing, compact disks, thermal paper, paper coatings and developer in dyes (Markey *et al.*, 2002). BPA derivates including tetrabromobisphenol A (TBBPA) and tetrachlorobisphenol A (TCBPA) are used as flame retardants for building materials and plastic products including epoxy resin electronic circuit boards (Markey *et al.*, 2001).

BPA alters ovarian function through its action as an EDC. *In vitro* exposure of rat ovarian theca-interstitial and granulosa cells to BPA increased testosterone, pregnenolone and E_2 production. Also there were increased mRNA levels of *Cypc17*, *Cyp19*, *Cyp11a* and *StAR* and decreased P_4 levels with BPA exposure (Zhou et al., 2008). BPA can bind to both E_2 receptors (ERα and ERβ) and induce E_2-dependent gene expression including *Cyp1b1* in rats (Naciff *et al.*, 2002). PND1 female rats exposed to BPA (50 ug/kg/day; subcutaneous injection; s.c.) demonstrated premature puberty onset and anestrus (Adewale *et al.*, 2009). Also, BPA increases initiation of primordial follicle recruitment from PND 1–7, subsequently reducing the primordial follicle number (Rodriguez *et al.*, 2010). Neonatal BPA exposure from PND 1–10 (500 μg/kg/d) resulted in anovulation and infertility in female rats from 4 months of age onwards. These female rats also had an accelerated GnRH pulse frequency in hypothalamic explants (Fernandez *et al.*, 2010), suggesting an effect of BPA on the hypothalamic-pituitary-gonadal (HPG) axis. BPA exposure also affects the ovary in adulthood following *in utero* exposure. Dams exposed to BPA (250 ng/kg/day) from GD 9 to 21 resulted in the appearance of blood-filled ovarian bursa in female offspring at 6 month of age (Markey *et al.*, 2003).

Little is known about how BPA is metabolized in the ovary and whether such metabolism impacts BPA-induced ovotoxicity. An increase in GSTM expression in gonads of adult fish

has been demonstrated during BPA exposure (600 μg/L; Yu *et al.*, 2008), indicating that GSH conjugation catalyzed by GSTM may be a metabolism route for BPA.

Phthalates

Phthalates are diesters of phthalic acid mostly used as plasticizers in polyvinyl chloride products (DHHS., 2005). Phthalates are ubiquitous environmental toxicants to which million people are exposed daily and human phthalate exposure has been confirmed by detection of phthalate metabolites in urine (Blount *et al.*, 2000a; Blount *et al.*, 2000b). Around 18 billion pounds of phthalates are used in plastic product manufacture annually (Blount *et al.*, 2000a), and the most commonly used phthalates are di-2-ethylhexyphthalate (DEHP), dibutyl phthalate (DBP) and diethyl phthalate (DEP).

Phthalates esters are E_2 analogues and have estrogenic effects (Sonnenschein and Soto, 1998). DEHP causes ovarian toxicity in rodents (Reddy and Lalwai, 1983; Lake *et al.*, 1987; Davis *et al.*, 1994a). Female mice and rats exposed to DEHP demonstrate reduced implantations, increased fetus resorption, decreased fetal weights and malformations (Kaul *et al.*, 1982; Lovekamp-Swan and Davis, 2003). DEHP (1–100 ml/kg; 1, 5, 10 d; s.c.) exposure in mice resulted in decreased pregnancy rates. Also, decreased growing follicle and corpora lutea numbers were observed (Agarwal *et al.*, 1989). At higher doses, DEHP exposure (500–3000 mg/kg/day; gavage) in mice and rats delayed vaginal opening with prolonged estrous cycles (Grande *et al.*, 2006), altered ovulation (Davis *et al.*, 1994a), and decreased fertility (Lamb *et al.*, 1987; Gray *et al.*, 2006). The impact of DEHP on anovulation may due to defects in HPG axis signaling (Svechnikova *et al.*, 2007) or may also be attributable to decreased E_2 production. DEHP exposure (100 μM for 24 h) decreased production of E_2 in cultured rat granulosa cells (Treinen *et al.*, 1990).

The toxic effects of DEHP are mostly carried out by its active metabolite mono-(2-ethylhexyl) phthalate (MEHP). MEHP acts by activating peroxisome proliferator-activated receptors (PPARs), the main regulators for lipid metabolism and cell differentiation (Maloney and Waxman, 1999). All known isoforms of PPAR (α, β and γ) are expressed in the rat ovary (Braissant *et al.*, 1996), where PPAR α and β are located in theca cells and stromal tissue and PPARγ is highly expressed in pre-ovulatory granulosa cells (Komar *et al.*, 2001). MEHP (0–400 μM) decreases E_2 production in a dose-dependent manner in cultured rat granulosa cells (Davis *et al.*, 1994b), likely due to decreased *Cyp19* transcription and increased mRNA expression of *Hsd17b1* in cultured rat granulosa cells (200 μM MEHP; 48 h; Lovekamp and Davis, 2001). The observed decreased *Cyp19* mRNA expression is mediated through the action of both PPAR α and γ receptors (Lovekamp-Swan and Davis, 2003).

Chemotherapeutics

Anti-neoplastic therapy destroys ovarian follicles and consequently predisposes women to infertility and premature menopause (Absolom *et al.*, 2008). Increased survival rates (Blumenfeld *et al.*, 1996; Maltaris *et al.*, 2006) over the past few decades have raised the reproductive toxicity consequences of chemotherapeutic agents as a serious issue. Cyclophosphamide (CPA) is used to treat childhood leukemia, Hodgkin's and non-Hodgkin's lymphomas, and breast cancer, bone and tissue sarcomas in adults (Colvin, 1999; Hurley, 2002; Chemaitilly *et al.*, 2006). It is also used as an immunosuppressant for multiple sclerosis and organ transplant rejection (Colvin, 1999).

In hepatic tissue, CPA undergoes activation by CYP2B1 and CYP3A4 to 4-hydroxycyclophosphamide (4-HC) that is converted through non-enzymatic reactions to form aldophosphamide (AP), and subsequently phosphoramide mustard (PM) and acrolein

(Ludeman, 1999). PM has been demonstrated to be the ultimate ovotoxic CPA metabolite (Plowchalk and Mattison, 1991; Desmeules and Devine, 2006). CPA can also be converted to carboxyphosphamide (CPM) or 4-ketocyclophosphamide (4-KTCP), which are nontoxic CPA metabolites due to their inability to form PM.

Ovarian follicles are the principal targets of CPA-induced ovotoxicity (Waxman, 1983). Sprague-Dawley female rats exposed to CPA (500 mg/kg; i.p.) demonstrate reduced ovarian follicle numbers in a time- and dose-dependent manner. Complete destruction of primordial follicles was observed within 3 d of CPA exposure (Shiromizu *et al.*, 1984). Mouse primordial follicles are more susceptible than those of rats to CPA-induced depletion. Almost complete (98%) destruction of primordial follicles occurred when female mice were given a single CPA exposure (75 mg/kg; i.p; Plowchalk and Mattison, 1991).

Granulosa cells of large pre-antral and antral follicles are also substantially damaged by CPA treatment in mice and rats (Lopez and Luderer, 2004; Desmeules and Devine, 2006). Induction of the mitochondrial apoptotic pathway has been demonstrated in granulosa cells of secondary and antral follicles of rats following a single CPA exposure with a significant decrease in ovarian GSH level (300 mg/kg; i.p.; Lopez and Luderer, 2004). GSH has the capacity to detoxify reactive oxygen species (ROS; Dalton et al., 2004), however, no additional follicle loss was achieved however when GSH was suppressed using buthionine sulfoximine (BSO; 5 mmol/kg; i.p.) during CPA exposure (Lopez and Luderer, 2004). Additionally, human granulosa tumor cells, COV434, treated with a CPA metabolite, 4-hydroxycyclophosphamide (4-HC, 50 μM) had increased oxidative stress and reduction of GSH to oxidized GSH (GSSG; Tsai-Turton *et al.*, 2007b). Similar to that of DMBA, use of TAM protects against CPA-induced primordial follicle loss (Ting and Petroff, 2010). These studies suggest that oxidative stress is involved in the apoptotic pathway induced by CPA and that GSH conjugation is a potential ovarian detoxification route.

Cultured PND4 mouse ovaries exposed to PM (3 μM) had rapid depletion of primordial oocytes and granulosa cells of larger follicles (Desmeules and Devine, 2006). Positive TUNEL staining of pyknotic granulosa cells was demonstrated, however, no increase in caspase-3 activation was observed (Desmeules and Devine, 2006). PM destroys tumor cells by binding covalently to DNA, inducing DNA-DNA and DNA-protein crosslinks and DNA double-strand breaks (DSB; (Colvin *et al.*, 1999; Hurley *et al.*, 2002; Helleday *et al.*, 2008) and this mode of action has been confirmed in ovarian oocytes (Petrillo *et al.*, 2011). Phosphorylation of H2AX (γH2AX) occurs in response to DSB and recruits DNA repair protein to the DSB sites (Rogakou *et al.*, 1998; Paull *et al.*, 2000; Modesti and Kanaar, 2001). γH2AX expression was observed within 9 h after PM (3–10 μM) exposure in cultured PND4 mouse and rat ovaries supporting that DSB were being induced by PM exposure (Petrillo *et al.*, 2011). Interestingly, the DSB were detected at concentrations at which follicle loss was not observed, suggesting that germline DNA damage could occur prior to evidence of follicle depletion (Petrillo *et al.*, 2011).

Aging

In mammals, ovarian aging is characterized by the reduction in ovarian follicle number and oocyte quality as well as dysfunction at the hypothalamic-pituitary-gonadal axis, culminating in reproductive senescence (Labhsetwar, 1967; Butcher and Page, 1981; Nozaki *et al.*, 1995; Brann DW, 2005). Aging has also been associated with increased ROS generation and decreased antioxidant protection ultimately leading to a wide range of cellular damage (Dean *et al.*, 1993). Ovarian cells are not an exception to this; there is increased ROS and decreased antioxidant levels in oocytes, cumulus cells as well as in follicular fluid of older women (45 years) undergoing assisted reproduction (Wiener-Megnazi *et al.*, 2004; Tatone *et al.*, 2006). GSH concentrations decreased in aged mouse

oocytes compared to those from young mice (Hamatani *et al.*, 2004; Brink *et al.*, 2009), while superoxide dismutase (SOD) and glutathione peroxidase (GPX) enzymatic activities decreased in ovaries from postmenopausal compared to premenopausal women (Okatani *et al.*, 1993). Furthermore, age-related increases in oxidative lipid, protein and DNA damage in ovarian interstitial cells and follicles have been reported with a decrease in glutaredoxin 1 (*Glrx1)* and *Gstm2* and an increase in *Gpx1* antioxidant gene expression (Lim and Luderer, 2010). Thus, due to the decrease in the ovarian protective response with aging, greater potential for damage caused by ovotoxicants is likely as females approach menopause.

In summary, ovarian metabolism plays an important role in determination of the ovotoxic impacts of a chemical exposure, and the studies on TCE, DMBA and VCD have begun to unravel mechanisms involved in xenobiotic metabolism. While insights on the impact of MXC, TCDD, BPA, Phthalates, and anti-neoplastic agents on normal ovarian processes are being delineated, there remains a dearth of information on whether and how these parent compounds are transformed to more active compound(s) in the ovary. Additionally, how these chemicals and their metabolites are detoxified and eliminated from the body remains poorly understood. Until greater insight is gained on ovarian xenobiotic biotransformation processes, attempts to develop therapeutic strategies to prevent follicle depletion and disruption to normal ovarian physiological processes will be limited.

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Highlights

- Summary of ovotoxicant action during ovotoxicity
- Discussion of impact of biotransformation on chemical toxicity
- Identification of knowledge gaps in chemical metabolism

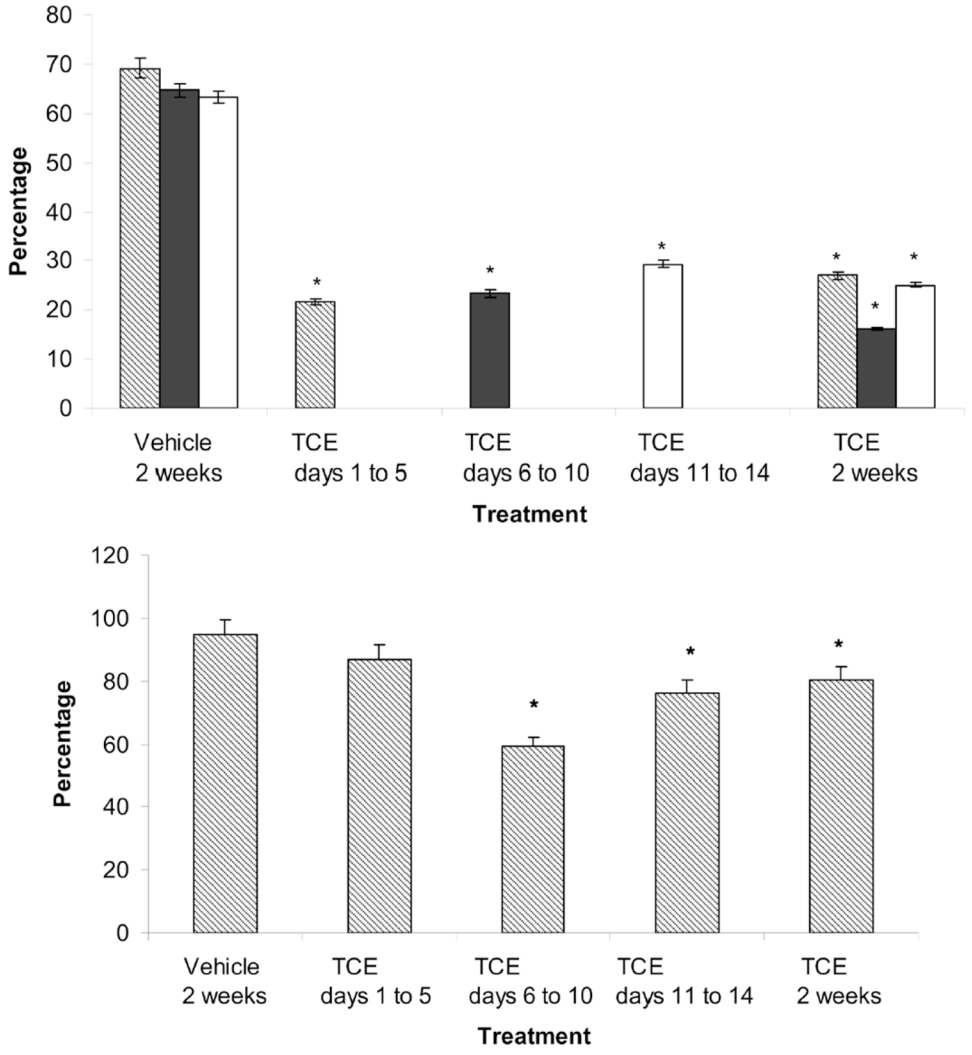

Figure 1.

(A) Decreased fertilizability of oocytes from TCE-exposed (0.45% TCE (v/v) in 3% Tween) rats compared with oocytes from vehicle-control (3% Tween) rats. Fertilization was assessed by the presence of decondensed sperm heads. Values represent the least squares means from three replicates. * indicates $P < 0.05$ compared with the vehicle control. **(B) Oocytes with bound sperm.** Bars represent the percentage of total oocytes (fertilized and unfertilized) that bound sperm. Oocytes from females treated with TCE on d 6 to 10, d 11 to 14 and for 2 wks bound fewer sperm compared with vehicle-controls. * indicates $P < 0.05$ compared with the vehicle control (Adapted from Wu and Berger, 2007 with copyright permission).

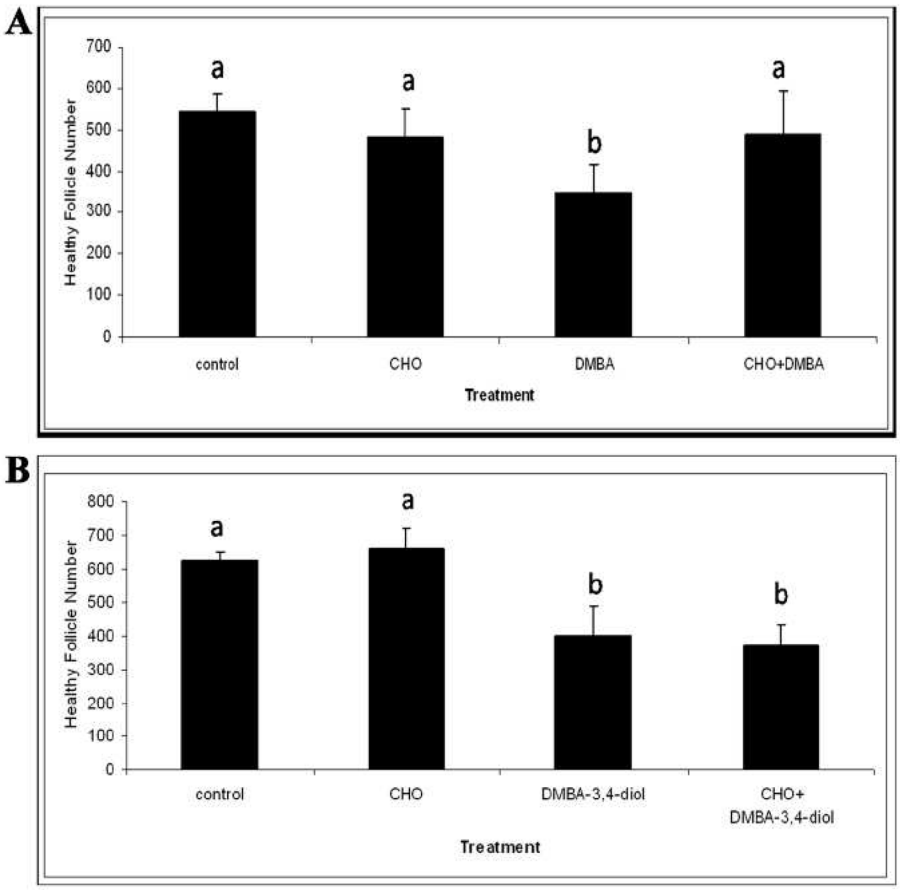

Figure 2. Effect of CHO (mEH inhibitor) on DMBA- or DMBA-3,4-diol-induced follicle loss

Ovaries from PND4 Fischer 344 rats were cultured with (A) vehicle control or media containing DMBA (1 μM) ± CHO (2 mM) for 4 d; or (B) vehicle control or media containing DMBA-3,4-diol (75 nM) ± CHO (2 mM) for 4 d. Following incubation, ovaries were collected, processed for histological evaluation and healthy follicles were counted. Values are mean ± SE total follicles counted/ovary, n=5. Different letters indicate significant difference between follicle number (Adapted from Igawa *et al.*, 2009 with copyright permission).

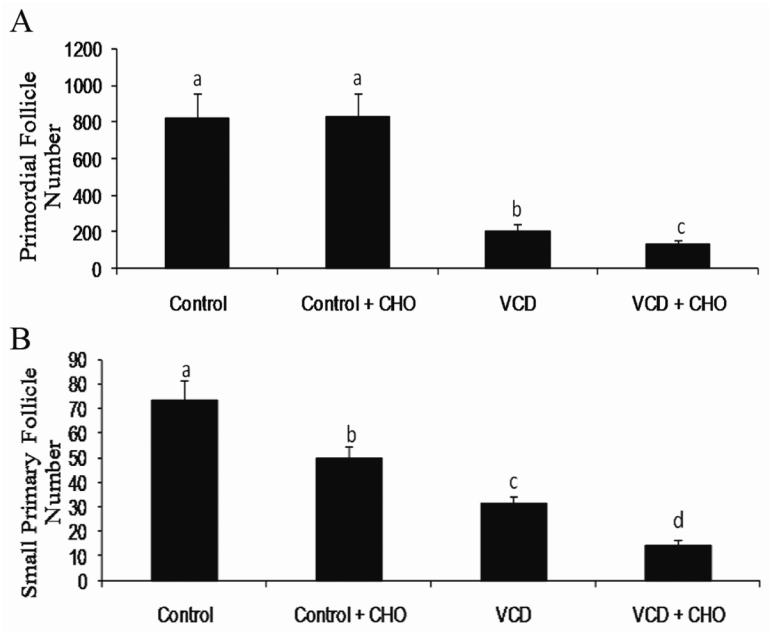

Figure 3. Effect of mEH inhibition on VCD-induced follicle loss

PND4 F344 rat ovaries were cultured in media containing vehicle control or VCD (30 μM), ± CHO (2 mM) for 8 d. Ovaries were processed for histological evaluation and healthy follicles were classified and counted as described in methods. Values are expressed as mean ± SE total follicles counted/ovary, n=5. Different letters indicate significant difference; $P < 0.05$ (Adapted from Bhattacharya *et al.*, 2012 with copyright permission).

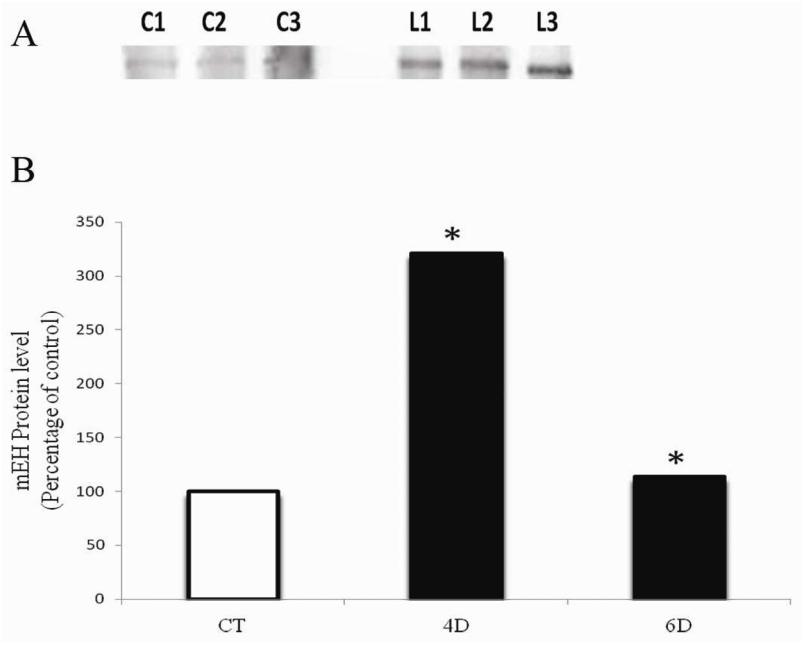

Figure 4. Temporal effect of PI3K inhibition on mEH protein

PND4 F344 rat ovaries were cultured in media containing vehicle control (Agency), ± 20 μM LY294002 for 4 or 6 d. Total protein was isolated and Western blotting was performed to detect mEH protein. (A) Representative Western blot is shown on day 4; Control = C; LY294002 = L. (B) Values are expressed as a percentage of control mean ± SE; n=3 (10 ovaries per pool). * $P < 0.05$; different from control (Adapted from Bhattacharya *et al.*, 2012 with copyright permission).